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Oleate desaturase enzymes of soybean: evidence of regulation through differential stability and phosphorylation

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Summary

The endoplasmic reticulum-associated oleate desaturase FAD2 (1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine Δ12-desaturase) is the key enzyme responsible for the production of linoleic acid in non-photosynthetic tissues of plants. Little is known, however, concerning the post-transcriptional mechanisms that regulate the activity of this important enzyme. The soybean genome possesses two seed-specific isoforms of FAD2, designated FAD2-1A and FAD2-1B, which differ at only 24 amino acid residues. Expression studies in yeast revealed that the FAD2-1A isoform is more unstable than FAD2-1B, particularly when cultures were maintained at elevated growth temperatures. Analysis of chimeric FAD2-1 constructs led to the identification of two domains that appear to be important in mediating the temperature-dependent instability of the FAD2-1A isoform. The enhanced degradation of FAD2-1A at high growth temperatures was partially abrogated by treating the cultures with the 26S proteasome-specific inhibitor MG132, and by expressing the FAD2-1A cDNA in yeast strains devoid of certain ubiquitin-conjugating activities, suggesting a role for ubiquitination and the 26S proteasome in protein turnover. In addition, phosphorylation state-specific antipeptide antibodies demonstrated that the Serine-185 of FAD2-1 sequences is phosphorylated during soybean seed development. Expression studies of phosphopeptide mimic mutations in yeast suggest that phosphorylation may downregulate enzyme activity. Collectively, the results show that post-translational regulatory mechanisms are likely to play an important role in modulating FAD2-1 enzyme activities.

Keywords: oleate desaturase, FAD2, protein phosphorylation, 26S proteasome, polyunsaturated fatty acids, soybean (*Glycine max*).

Introduction

The temperature-dependent alteration of cellular fatty acid composition is a phenomenon commonly observed in nature. In organisms that produce polyunsaturated fatty acids (PUFAs), there is typically an inverse relationship between growth temperature and PUFA accumulation in membrane lipids: growth at low temperatures leads to an increase in the ratio of PUFAs to unsaturated and monounsaturated fatty acids (Neidleman, 1987; Thompson, 1993). Temperature-mediated fluctuations in PUFA content within cellular membranes are believed to help maintain suitable levels of membrane fluidity, and thus assist in the survival of the cell across a wide range of growth temperatures.

The same inverse relationship between PUFA accumulation and temperature is also observed in the seed-storage lipids of commercial oilseeds such as soybean, sunflower and canola. In soybean, growth at elevated temperatures coincides with a decrease in the linoleic ($18{:}2^{\Delta 9,12}$) and linolenic ($18{:}3^{\Delta 9,12,15}$) acid concentrations of seed triacylglycerols and a corresponding increase in oleic acid ($18{:}1^{\Delta 9}$) levels (Carver *et al.*, 1986; Rebetzke *et al.*, 1996; Rennie and Tanner, 1989). The relative quantities of the various saturated and unsaturated fatty acids are critical factors that influence the quality and commercial applications of plant oils. Understanding the mechanisms by which fatty acid composition is determined during seed

development under varying environmental conditions would therefore represent an important step towards the ultimate goal of regulating these processes in a directed, predictable manner.

Investigations into the mechanisms of temperature-dependent alterations of fatty acid composition of plant membrane lipids have provided evidence of control at both transcriptional and post-transcriptional levels for genes encoding the ω-3 desaturases responsible for converting linoleic acid to linolenic acid. Transcript accumulation of a gene encoding a plastid-localized ω-3 desaturase in maize was shown to increase in response to low growth temperature, suggesting regulation at the level of gene transcription (Berberich *et al.*, 1998). Investigations of a microsomal membrane-associated ω-3 desaturase in wheat, however, revealed an enhancement in enzyme accumulation and linolenic acid production at low growth temperatures in the absence of notable changes in transcript abundance (Horiguchi *et al.*, 2000). A recent study of the two plastidial ω-3 desaturases found in Arabidopsis (FAD7 and FAD8) demonstrated the importance of both transcriptional and post-transcriptional mechanisms in regulating the trienoic fatty acid levels of the leaf. It has long been recognized that *FAD8* transcript levels decrease dramatically in response to growth at elevated temperatures, whereas *FAD7* mRNA levels remain unchanged (Gibson *et al.*, 1994). By constructing and testing a series of chimeric *FAD7:FAD8* genes, Matsuda *et al.* (2005) found that the C-terminal 44 aa of the FAD8 enzyme specifically destabilize the protein at high temperatures, even in the absence of reduced transcript accumulation.

Evidence of post-transcriptional control has also been implicated in the temperature-dependent regulation of the *FAD2*-encoded ω-6 desaturase enzymes responsible for conversion of oleic acid to linoleic acid in the endoplasmic reticulum. Although a functional *FAD2* gene has been shown to be required for Arabidopsis growth and survival at low temperatures (Miquel *et al.*, 1993), no change in steady-state *FAD2* transcript levels was observed when plants were transferred from 22 to 6°C over a 3-day time course (Okuley *et al.*, 1994). In soybean, two different *FAD2* isoforms have been reported: a constitutively expressed gene designated *FAD2-2*, and a seed-specific gene designated *FAD2-1*; the latter gene plays the predominant role in determining the PUFA content of the seed-storage oil (Heppard *et al.*, 1996; Kinney, 1997). Despite notable increases in PUFA accumulation in seed triacylglyerols and leaf membrane lipids as soybean plants were grown over a range of decreasing growth temperatures, steady-state transcript levels of *FAD2-1* and *FAD2-2* remained constant, and at the highest growth temperature were even shown to be elevated (Heppard *et al.*, 1996).

We found that the soybean genome actually encodes two closely related, actively expressed *FAD2-1* isoforms. Expression analyses in yeast of these two isoforms, designated *FAD2-1A* and *FAD2-1B*, unexpectedly revealed major differences in the activities and/or stabilities of the two closely related enzymes, particularly in response to growth at elevated temperatures. In addition, protein-blotting assays using an oligopeptide-based antibody generated against a putative phosphorylation motif suggested that the soybean FAD2-1 enzymes can be phosphorylated. The potential roles of enzyme phosphorylation and temperature-dependent regulation of protein turnover of FAD2-1 enzyme activities during seed development are discussed.

Results

Identification of two closely related FAD2-1 *genes in soybean*

As part of ongoing efforts to understand the molecular regulation of storage oil biosynthesis in soybean, we utilized a PCR-based approach to isolate *FAD2-1* cDNAs using RNAs isolated from developing soybean seeds as a template. This approach led to the isolation of two closely related, yet distinct *FAD2-1* cDNAs that we designated *FAD2-1A* and *FAD2-1B*. *FAD2-1A* is identical to the sequence reported by Heppard *et al.* (1996); GenBank accession no. L43920). The *FAD2-1B* sequence is 93.8% identical to the DNA sequence of *FAD2-1A*, and the predicted protein sequences are also 93.8% identical, differing at only 24 positions (asterisks in Figure 1a). *FAD2-1A* and *FAD2-1B* are both approximately 67% identical to the constitutively expressed *FAD2-2* DNA sequence (data not shown). None of the amino acid changes observed between FAD2-1A and FAD2-1B alters the predicted topological model of these enzymes within the endoplasmic reticulum membrane (Figure 1b).

More than 286 000 expressed sequence tags (ESTs) derived from 80 cDNA libraries, representing a wide variety of soybean tissue types and developmental stages, have been deposited in public databases (Shoemaker *et al.*, 2002). Analysis of BLASTN results using *FAD2-1A* and *FAD2-1B* as query sequences enabled us to categorize 12 ESTs as corresponding to *FAD2-1A* and 30 ESTs as corresponding to *FAD2-1B*. As shown in Table 1, all *FAD2-1* ESTs were recovered from libraries corresponding to tissues and/or developmental stages associated with seed development. In contrast, over 100 ESTs corresponding to *FAD2-2* cDNAs are found in GenBank, the great majority of which were isolated from non-seed libraries (data not shown). These results are consistent with the study of Heppard *et al.* (1996) who concluded that *FAD2-1* and *FAD2-2* represent seed-specific and constitutive ω-6 desaturase genes, respectively. The *in silico* analysis of the *FAD2-1A* and *FAD2-1B* ESTs suggests that both isoforms are actively expressed during soybean seed development. The *FAD2-1B* gene, however, is likely to

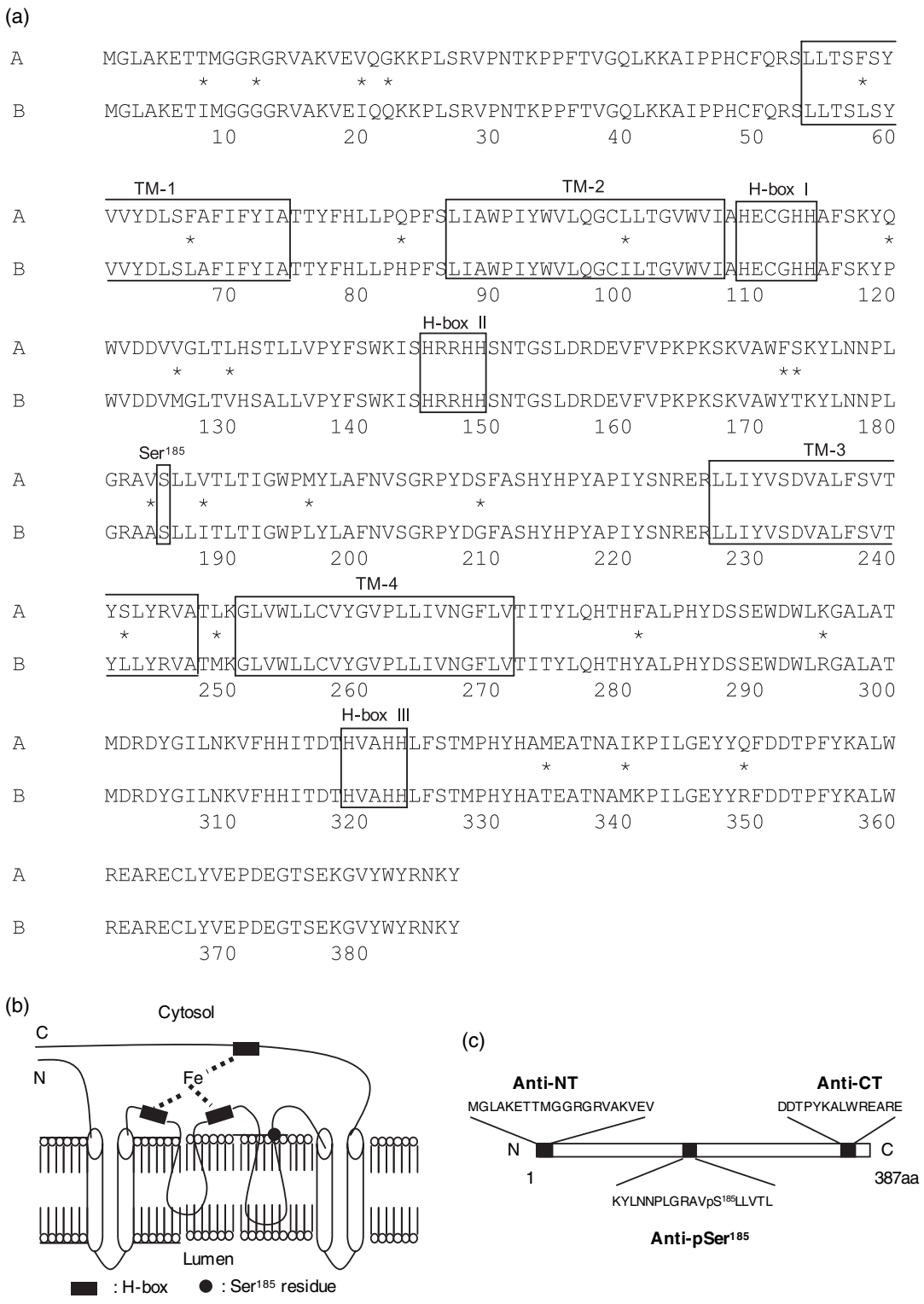

Figure 1. The FAD2–1 enzymes of soybean.
(a) Sequence alignment of FAD2-1A (A) and FAD2-1B (B). Amino acid residues that differ between the two sequences are indicated by an asterisk. Predicted transmembrane (TM) domains and histidine box (H-box) motifs are boxed. The Ser residue that serves as a substrate for phosphorylation is also indicated.
(b) Putative topology of FAD2-1 enzymes. The model depicted was derived from Dyer and Mullen (2001); Dyer *et al.* (2002); Shanklin *et al.* (1994).
(c) Schematic diagram showing the peptide sequences used to generate the amino-terminal, carboxy-terminal and pSer^{185} FAD2-1 antibodies (Anti-NT, Anti-CT and Anti-pSer^{185}, respectively).

Table 1 Number and tissue source of FAD2-1A- and FAD2-1B-specific ESTs

Source of library	FAD2-1A ESTs	FAD2-1B ESTs
Immature cotyledons (20–50 mg seed)	0	7
Immature cotyledons (100–300 mg seed)	8	19
Pre-senescence mature seed pods	3	2
Somatic embryos	1	0
Immature whole pods (2 cm long)	0	1
Immature seed coat (200–300 mg seed)	0	1
Total	12	30

be more highly represented than the *FAD2-1A* isoform in the mRNA pools of the developing seed.

Expression of soybean FAD2-1 *genes in yeast*

To initiate an investigation of the structural and functional properties of the two seed-specific microsomal oleate desaturases of soybean, the *FAD2-1A* and *FAD2-1B* cDNAs were cloned downstream of the constitutively active Adh1 promoter in the pDB20 yeast expression vector (Becker *et al.*, 1991) and transformed into wild-type yeast strain CTY182. As an additional tool, antibodies were generated against oligopeptides corresponding to the N-terminal 20-aa (Anti-NT) and C-terminal 14-aa residues (Anti-CT) of the predicted protein sequence of *FAD2-1A* (Figure 1c). The 14 residues used to generate Anti-CT are identical between the *FAD2-1A* and *FAD2-1B* predicted products, whereas three residues differ in the first 20 aa at the N-termini of the predicted proteins. Because the three differences at the N-terminus resulted in much weaker binding of the Anti-NT antibody to FAD2-1B than to FAD2-1A (data not shown), the Anti-CT antibody was used in all experiments where the two soybean FAD2-1 enzymes were directly compared. In addition to the FAD2-1 proteins, one or two slightly more highly migrating proteins commonly appeared on immunoblots when the Anti-CT antibody was used as a probe. The visualization of these proteins in yeast cells expressing the empty pDB20 vector alone, however, demonstrated that they are unrelated to the FAD2-1 sequence (Figure 2a).

In yeast, alterations in membrane fluidity in response to growth temperature can be regulated not only through changes in levels of fatty acid desaturation, but also by adjusting the relative ratios of C_{16} and C_{18} fatty acids (Okuyama and Saito, 1979). For yeast strain CTY182, we observed no significant changes in fatty acid chain length between cultures grown at 30 versus 20°C, but consistently observed a modest increase in the monounsaturated (16:1 + 18:1) fatty acid content in cells grown at the lower temperature. On average, 73% of the total fatty acid pool was monounsaturated in cultures grown at 30°C compared with 78% at the 20°C growth temperature (data not shown).

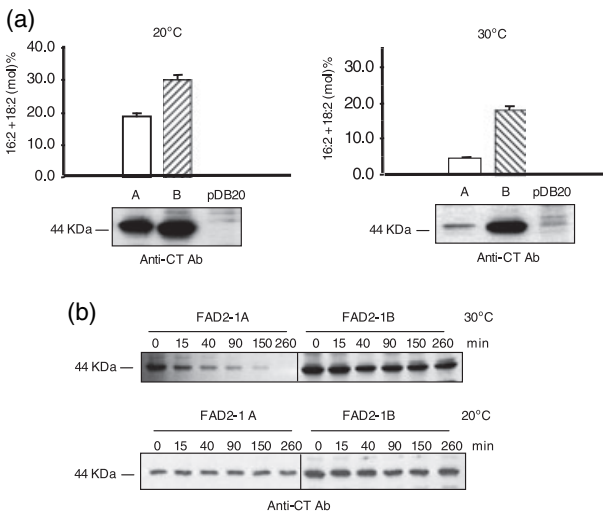

Figure 2. Expression of FAD2-1 enzymes in yeast.
(a) Dienoic fatty acid content and immunoblot analysis of yeast cells expressing FAD2-1A (A), FAD2-1B (B) and a vector-only control (pDB20). Error bars, SD ($n = 3$).
(b) Immunoblot analysis of FAD2-1 accumulation in response to cycloheximide treatment. Aliquots of yeast cultures were collected at the designated time points after cycloheximide addition. The immunoblot corresponding to FAD2-1A at 30°C was exposed for longer than the corresponding FAD2-1B blot to allow visualization of this less-abundant protein.

CTY182 cells were transformed with the soybean *FAD2-1* constructs, cultured at 20 and 30°C, and assayed for fatty acid composition. As shown in Figure 2(a), dienoic fatty acids (16:2 + 18:2) constituted approximately 18% of the total fatty acid pool when yeast cells transformed with *FAD2-1A* were grown at 20°C. The *FAD2-1B* construct mediated even higher levels of dienoic fatty acid accumulation (approximately 30%) under the same growth conditions. The relative differential between FAD2-1A and -1B was even more dramatic at the 30°C growth temperature. Although the elevated growth temperature led to a reduction in the dienoic fatty acid levels mediated by both constructs, the decrease was much greater in the *FAD2-1A*-transformed yeast. The 16:2 + 18:2 content was reduced over fourfold in the cells expressing *FAD2-1A* at 30 versus 20°C, compared with a more modest 40% decrease in cells transformed with *FAD2-1B* (Figure 2a). This result was confirmed in numerous independent experiments.

Differences in dienoic fatty acid accumulation between the *FAD2-1A*- and *FAD2-1B*-expressing yeast cells are probably attributable to either differential transcript accumulation, differential enzyme activity, or differential enzyme stability. Differences in translational efficiencies were not considered likely because the *FAD2-1A* and *FAD2-1B* cDNAs were cloned into the pDB20 vector in a manner giving identical 5′ non-translated sequences upstream of the respective start methionine codons. Also, semi-quantitative PCR analysis of the soybean *FAD2-1* genes failed to reveal significant differences in transcript accumulation between

yeast cells expressing *FAD2-1A* versus *FAD2-1B* (data not shown). In contrast, protein-blotting assays revealed major differences in protein accumulation. Although FAD2-1B protein accumulation was somewhat higher than FAD2-1A at the 20°C growth temperature, the differential was markedly enhanced at 30°C (Figure 2a).

To further examine the relative stability of the FAD2-1A and FAD2-1B proteins in yeast, cycloheximide was used to block protein translation in a time-course experiment. Immunoblot analysis using the Anti-CT antibody revealed that the *in vivo* decay of FAD2-1A was much faster than FAD2-1B at the 30°C growth temperature (Figure 2b). Although FAD2-1B protein levels were consistently higher than FAD2-1A at the 20°C growth temperature, the differential was modest, and both proteins were relatively stable over the course of the experiment. The good overall correlation between FAD2-1 protein accumulation, as judged by immunoblot analysis, and the observed fatty acid profiles (Figure 2a) suggests that differences in enzyme stability are primarily responsible for the differing dienoic fatty acid levels maintained in *FAD2-1A*- versus *FAD2-1B*-transformed yeast cells.

Defining domains responsible for the temperature-associated stability of FAD2-1 enzymes

The 24 amino acid positions where the predicted FAD2-1A and FAD2-1B enzymes differ are dispersed throughout the proteins (Figure 1a). To assist in establishing the specific regions of the protein responsible for conferring stability (or instability) on the enzyme at high growth temperatures, chimeric constructs were generated that resulted in the sequential replacement of the N-terminal portions of the FAD2-1A protein with those present on the FAD2-1B isoform, and *vice versa* (Figure 3a,b). Immunoblot and fatty acid analyses were conducted on yeast cells expressing the resulting chimeric enzymes.

At the 30°C growth temperature, replacing the initial 44 aa of the FAD2-1B enzyme with those found in FAD2-1A (construct AF_1BS_1) led to a reduction in protein accumulation and dienoic fatty acid accumulation to levels similar to that observed with the wild-type FAD2-1A enzyme. The reciprocal replacement of this region from FAD2-1B onto the FAD2-1A enzyme (construct BF_1AS_1), however, failed to enhance the stability or dienoic fatty acid production

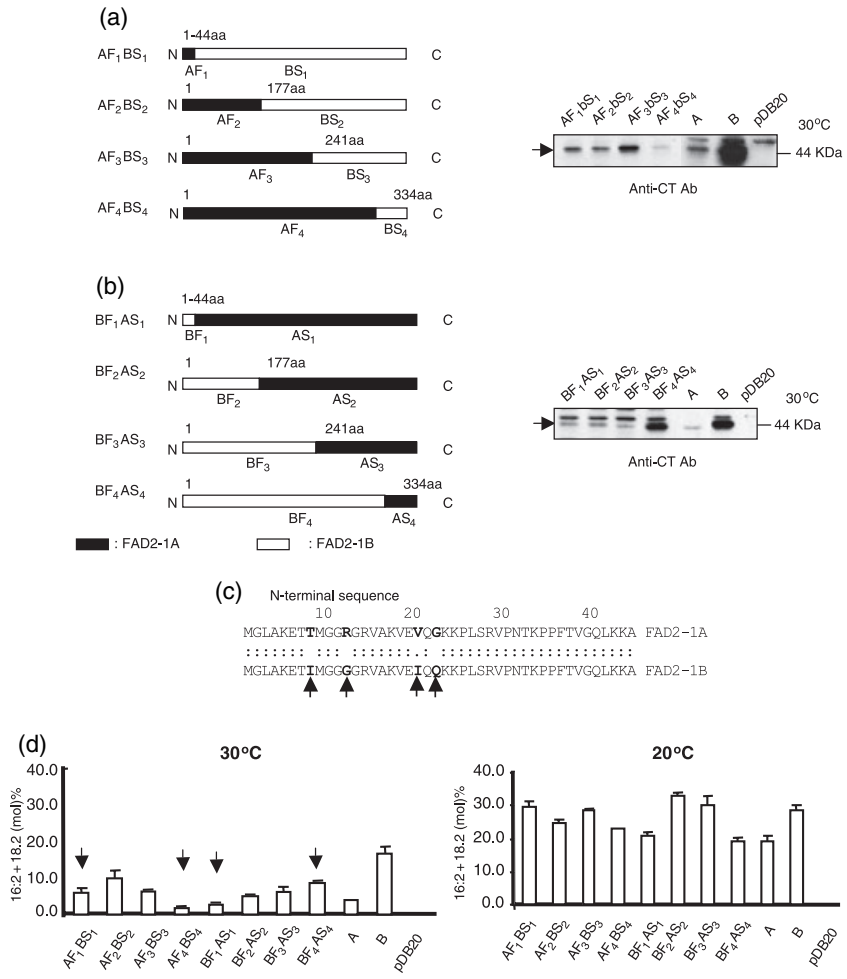

Figure 3. Expression of chimeric FAD2-1 proteins in yeast.
(a,b) Schematic diagrams showing the nested constructs AF_1BS_1, AF_2BS_2, AF_3BS_3, AF_4BS_4, BF_1AS_1, BF_2AS_2, BF_3AS_3 and BF_4AS_4 and their product accumulation profiles in yeast cells grown at 30°C. Black boxed regions represent portion of constructs derived from FAD2-1A; white boxed regions originated from FAD2-1B. Arrows indicate bands corresponding to recombinant FAD2-1 proteins.
(c) Sequence alignment of the N-terminal 44 aa of FAD2-1A and FAD2-1B. Arrows highlight the four residues that differ between the two isoforms in this region.
(d) Dienoic fatty acid content of yeast cultures expressing the nested chimeric constructs at 20 and 30°C. Arrows indicate constructs discussed in the text. Error bars, SD ($n = 3$).

mediated by FAD2-1A at the higher temperature. In either case, alterations in enzyme stability/activity appeared to be manifest only at the 30°C growth temperature, as dienoic fatty acid content mediated by the AF_1BS_1 chimera at 20°C was similar to the FAD2-1B construct, and BF_1AS_1 was similar to FAD2-1A at the lower temperature (Figure 3d). Thus, although only four amino acid residues differ between the N-terminal 44 aa of the two FAD2-1 enzymes (Figure 3c), these differences appear to influence enzyme stability/activity at 30°C, but not at 20°C.

The region between amino acids 241 and 334 also appeared to play a role in the differential stability of the two soybean FAD2-1 enzymes. The incorporation of this region from FAD2-1A into the AF_4BS_4 construct resulted in further destabilization of the protein at 30°C (and a concomitant reduction in dienoic fatty acid synthesis) in comparison with the AF_1BS_1, AF_2BS_2 and AF_3BS_3 constructs (Figure 3a,d). Conversely, the addition of the 241–334-aa region from FAD2-1B in construct BF_4AS_4 greatly enhanced protein accumulation at 30°C in comparison with the other constructs of this series. Although the dienoic fatty acid levels mediated by the BF_4AS_4 construct at 30°C were increased with regard to the wild-type FAD2-1A enzyme, they still fell short of those obtained with wild-type FAD2-1B.

To test further whether the four amino acid differences found in the 241–334-aa region of FAD2-1A and FAD2-1B specifically affect enzyme stability and/or activity, mosaic constructs were generated in which this segment from FAD2-1B was substituted within a FAD2-1A construct, and *vice versa*. As shown in Figure 4, the introduction of these four amino acid residues from FAD2-1B into the FAD2-1A enzyme did confer a modest increase in protein accumulation and dienoic fatty acid accumulation at 30°C. More dramatic, however, was the negative influence of these substitutions from FAD2-1A on the FAD2-1B enzyme. Dienoic fatty acid accumulation and protein abundance of the FAD2-1B enzyme were both reduced to levels typically observed with the wild-type FAD2-1A enzyme when the 241–334-aa region of the latter enzyme was introduced into the former.

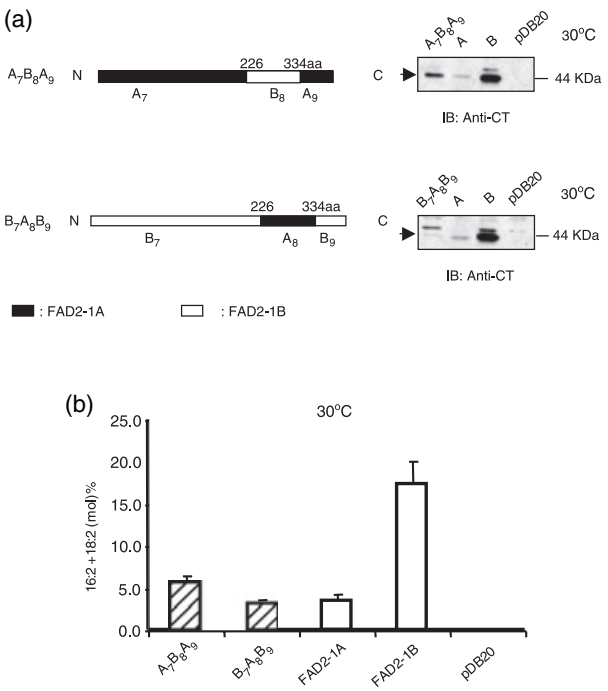

Figure 4. Expression of mosaic FAD2-1 constructs.
(a) Schematic diagram showing mosaic constructs $A_7B_8A_9$ and $B_7A_8B_9$ and their protein accumulation profiles in yeast cells grown at 30°C. Black boxed regions represent portion of constructs derived from FAD2-1A; white boxed regions originated from FAD2-1B. Arrows indicate band corresponding to recombinant FAD2-1 proteins.
(b) Dienoic fatty acid content of yeast cultures expressing the mosaic constructs shown in (a). Error bars, SD ($n = 3$).

Soybean FAD2-1 proteins are degraded in yeast through the ubiquitin/26S proteasome pathway

Extended exposure of our immunoblots routinely led to the visualization of higher molecular weight (HMW) species using the Anti-NT antibody probe generated against the N-terminal sequences of FAD2-1A. A typical example is shown in Figure 5(a). Because HMW ladders are characteristic of ubiquitin-conjugated proteins that are targeted for degradation via the 26S proteasome complex, we investigated this possibility by treating yeast cultures with the proteasome-specific inhibitor MG132 (Lim *et al.*, 2003). At 30°C, incubation of the growing yeast cultures with 10 μM MG132 for 3 h resulted in significantly enhanced protein accumulation for both FAD2-1A and FAD2-1B (Figure 5b). At the 20°C growth temperature, a modest increase in FAD2-1A levels was observed, whereas no change was apparent for the FAD2-1B enzyme. This result suggests that the 26S proteasome pathway is involved in FAD2-1 protein degradation and that the proteins are more susceptible to this mode of degradation at the elevated growth temperature.

In eukaryotic cells, ubiquitin conjugation catalysed by ubiquitin-conjugating enzymes (UBC-E2s) is an essential step in the 26S proteasome-degradation pathway. The selective degradation of target proteins through this pathway is determined through the interaction of the target protein with a specific E3 ligase and a compatible UBC E2 (Smalle and Vierstra, 2004). The yeast genome contains a total of 13 UBC E2 genes (http://www.yeastgenome.org), and knockout mutations in nine of these yield non-lethal phenotypes and are available in strain BY4741 (Open Biosystems, Huntsville, AL, USA).

FAD2-1A and *FAD2-1B* constructs were introduced into each of the nine viable UBC E2 deletion lines. Transformed yeast cells were grown at 20 and 30°C and assayed for fatty acid composition and FAD2-1 protein accumulation.

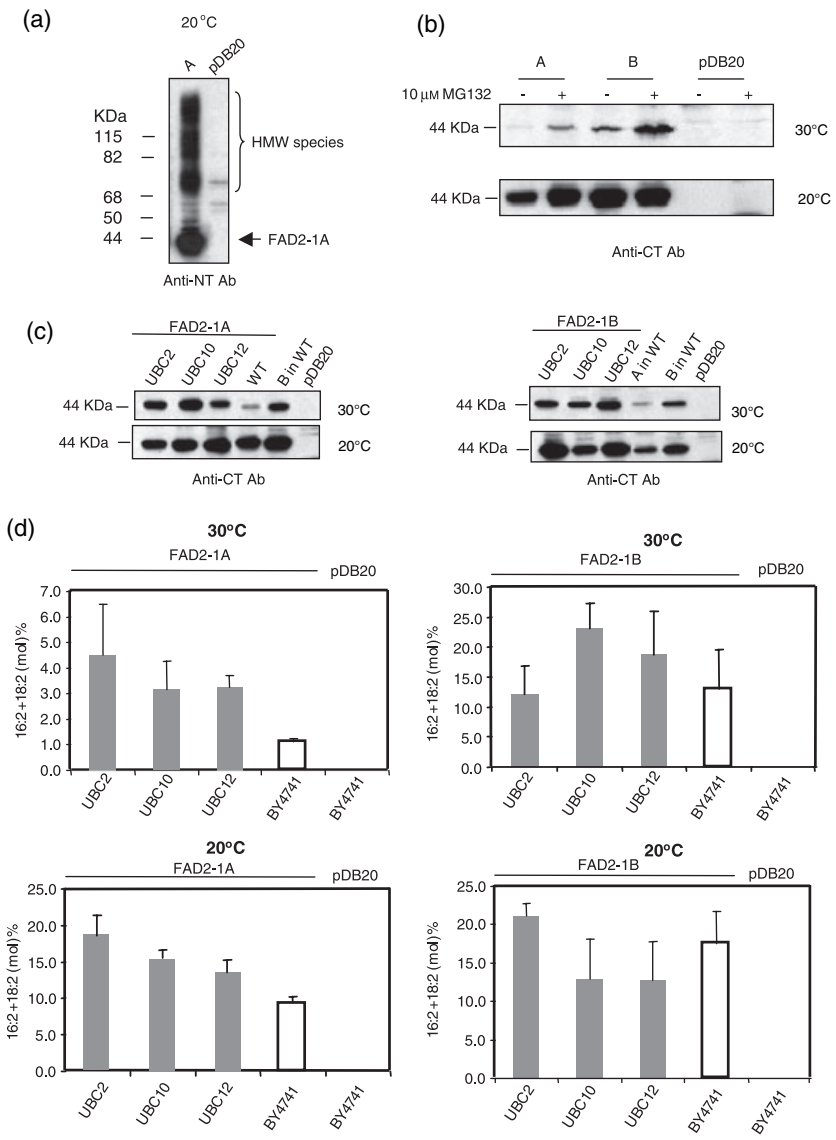

Figure 5. The ubiquitin/26S proteasome pathway is involved in the degradation of FAD2-1 proteins.
(a) High molecular weight (HMW) species observed in immunoblots using the Anti-NT antibody. Protein preparations from yeast cells expressing FAD2-1A (A) and the control pDB20 vector are represented.
(b) Immunoblot analysis of yeast cells expressing FAD2-1A (A) and FAD2-1B (B) in the presence and absence of the 26S proteasome inhibitor MG132 at 20 and 30°C.
(c) Immunoblot analysis of FAD2-1A and FAD2-1B expressed in UBC E2 deletion mutants and the wild-type yeast strain BY4741 at 20 and 30°C.
(d) Dienoic fatty acid content of the materials described in (c). Error bars, SD ($n = 3$).

Although the relative accumulation of FAD2-1A and FAD2-1B protein appeared to be similar in the wild-type BY4741 strain used in this experiment and the CTY182 strain used in previous experiments, we consistently observed less dienoic fatty acid accumulation using the BY4741 background (cf Figures 2a and 5d). Despite mediating less dienoic fatty acid accumulation than was obtained in CTY182, the 16:2 + 18:2 levels mediated by FAD2-1A in BY4741 at 20 and 30°C were comparable with those observed by Covello and Reed (1996) when they expressed the Arabidopsis *FAD2* gene in yeast strain DR1. Within the BY4741 yeast strain, an enhancement in dienoic fatty acid accumulation and FAD2-1A abundance was observed in three of the UBC E2 deletion lines. As shown in Figure 5(c), FAD2-1A accumulation at 30°C was significantly enhanced in yeast lacking functional *UBC2, UBC10* or *UBC12* genes. Enhancement of FAD2-1A levels was accompanied by a three- to fourfold increase in the 16:2 + 18:2 content of the cells (Figure 5d). Consistent with our previous results, alterations in fatty acid content and FAD2-1 protein accumulation were much more modest when the *FAD2-1B* construct was expressed in the *UBC10* and *UBC12* deletion lines. Interestingly, the *UBC2* deletion strain in which the greatest increases in dieonic fatty acid concentrations were observed with the *FAD2-1A* construct showed no significant differences in its fatty acid profile in comparison with the wild-type BY4741 yeast when transformed with the *FAD2-1B* gene.

Phosphorylation of FAD2-1 proteins

To date there have been no reports of the phosphorylation of oleate desaturase enzymes from any organism. *In silico*

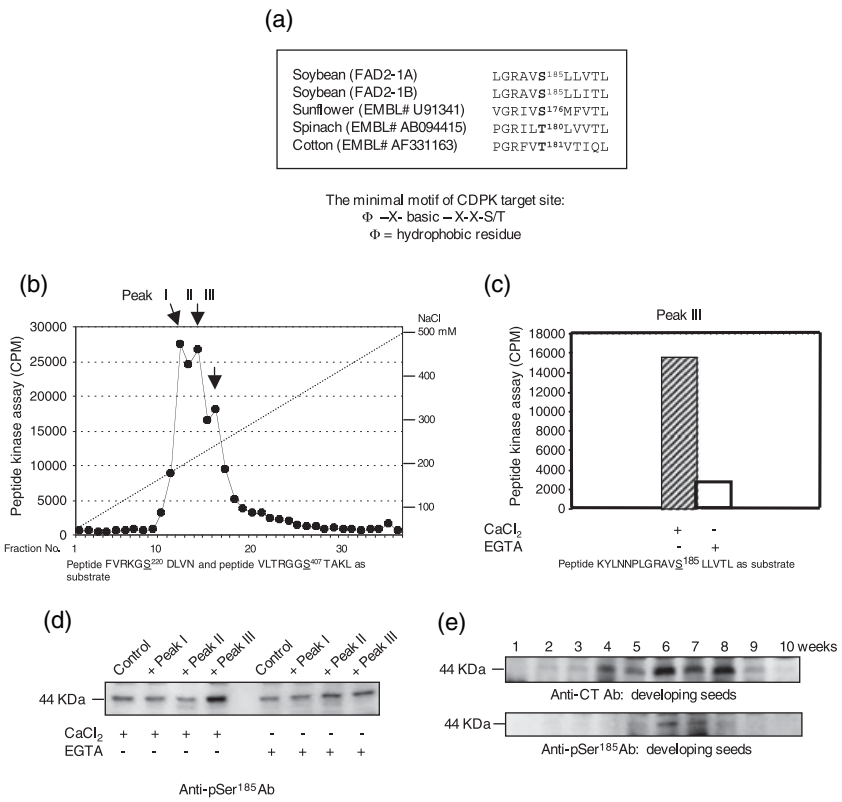

Figure 6. The Ser[185] residue is phosphorylated in developing soybean seeds.
(a) Alignment showing putative phosphorylation sites in FAD2 sequences of selected crop species. The residue serving as a potential CDPK substrate is indicated in bold.
(b) Peptide kinase activities recovered by anion-exchange chromatography. Peptide substrates utilized were the same as described by Tang *et al.* (2003).
(c) Peptide kinase assay using the cellular fraction corresponding to peak III from (b) and the Ser[185] oligopeptide as substrate. Assays were conducted in the presence of 0.1 mM $CaCl_2$ or 1 mM EGTA.
(d) Immunoblot detection by Anti-pSer[185] of soybean microsomal membrane preparations incubated with the peptide kinase fractions purified in (b).
(e) Immunoblot analysis of soybean microsomal membrane preparations using the Anti-CT and Anti-pSer[185] antibodies. Microsomes were isolated every 7 days throughout the course of seed development and maturation.

sequence analysis, however, suggested that Ser[185] of the soybean FAD2-1 proteins is located within a motif that could serve as a potential target for phosphorylation by calcium-dependent protein kinases (CDPKs; Huang and Huber, 2001). As shown in Figure 6(a), this motif is conserved among several plant oleate desaturases. To investigate the possibility that Ser[185] can be phosphorylated by plant protein kinases, a synthetic peptide was generated based on the predicted site and utilized as a substrate in peptide kinase assays.

Endogenous soybean protein kinase activities were partially purified from developing soybean seed extracts using anion-exchange chromatography. Initially, fractions possessing soybean CDPK activities were identified using peptide substrates that we have previously shown to be phosphorylated by this class of protein kinases in soybean (Figure 6b; Tang *et al.*, 2003). Peptide kinase assays were conducted by incubating the Ser[185] peptide substrate with the three fractions demonstrating peak protein kinase activities. Although some calcium-stimulated phosphorylation of the Ser[185] peptide substrate was observed in each of the three fractions tested, the most compelling activity was found in the fraction corresponding to peak III (Figure 6c).

To determine whether phosphorylation of the Ser[185] residue of FAD2-1 proteins occurs within the plant cell, an anti-phosphorylation state-specific antibody was generated against the phosphorylated version of the same Ser[185] oligopeptide sequence used in the protein kinase assays. The binding specificity of the pSer[185] anti-phosphopeptide antibody for phosphorylated antigen was >99:1 overbinding to the unphosphorylated peptide (as determined by ELISA assays conducted by the manufacturer). The Anti-pSer[185] antibody was used to probe immunoblots containing microsomal protein preparations isolated from developing soybean seeds (mid-maturation). As shown in Figure 6(d) (control lane), a 44-kDa protein was recognized by this antibody. When the microsomal preparations were pre-incubated with ATP, and one of the three peptide kinase fractions was collected via anion-exchange chromatography (Figure 6b), enhanced binding to the 44-kDa polypeptide was observed using fraction III supplemented with calcium. These results suggest that the Ser[185] residue of the FAD2-1 proteins was partially phosphorylated *in vivo*, and that phosphorylation *in vitro* could be mediated by a calcium-stimulated protein kinase (fraction III kinase).

Because FAD2-1 enzymes play such a pivotal role in determining the fatty acid composition of the seed-storage oil, we were interested in examining the pattern of enzyme phosphorylation throughout seed development. Using the Anti-CT antibody, FAD2-1 enzyme accumulation was notably enhanced 4 weeks after flowering, and continued to be elevated through the eighth week of seed development (Figure 6e). Using the Ser[185] anti-phosphopeptide antibody, a low level of binding was evident during the 6–8-week time

points. As the relative binding efficiencies of the Anti-CT and Ser[185] anti-phosphopeptide antibodies toward the FAD2-1 proteins are unknown, we cannot estimate what percentage of cellular FAD2-1 protein was phosphorylated. Nevertheless, these results support the hypothesis that some fraction of the FAD2-1 enzymes in soybean are indeed phosphorylated at Ser[185]. In contrast, no binding to FAD2-1 proteins was observed with the Anti-pSer[185] antibody using microsomal extracts from yeast cells expressing the FAD2-1 enzymes (data not shown). This result is not surprising, given that yeasts do not possess endogenous oleate desaturase enzymes and would therefore not be expected to encode protein kinase activities that would phosphorylate them.

Phosphorylation of Ser[185] may downregulate FAD2-1 activity

The phosphorylated state of Ser residues within a protein can often be mimicked by their replacement with the amino acids Asp or Glu (Kurland *et al.*, 1992; Wang *et al.*, 1992). As an initial step in exploring the potential role of Ser[185] phosphorylation, we used site-directed mutagenesis to create FAD2-1A constructs containing Asp and Glu residues at position 185. Furthermore, Ser[185] was replaced by the similarly sized Ala residue in a construct designed to create a protein that would be incapable of being phosphorylated at that site. The modified FAD2-1A constructs were expressed in yeast to assess the effects of the substitutions on enzyme activity and/or stability.

As shown in Figure 7, a slight decrease in dienoic fatty acid accumulation was observed in yeast cells expressing the FAD2-1A construct with the S185A substitution at both 20 and 30°C growth temperatures. A much more dramatic decrease in 16:2 + 18:2 content was observed when the Ser[185] residue was substituted with Asp or Glu. Protein blot analysis failed to distinguish significant differences in enzyme accumulation among the various constructs; therefore it is likely that enzyme activity *per se* is reduced in the phospho-Ser[185] mimic mutants. Similar results were obtained when the same mutations were introduced into the FAD2-1B construct and expressed in yeast (data not shown).

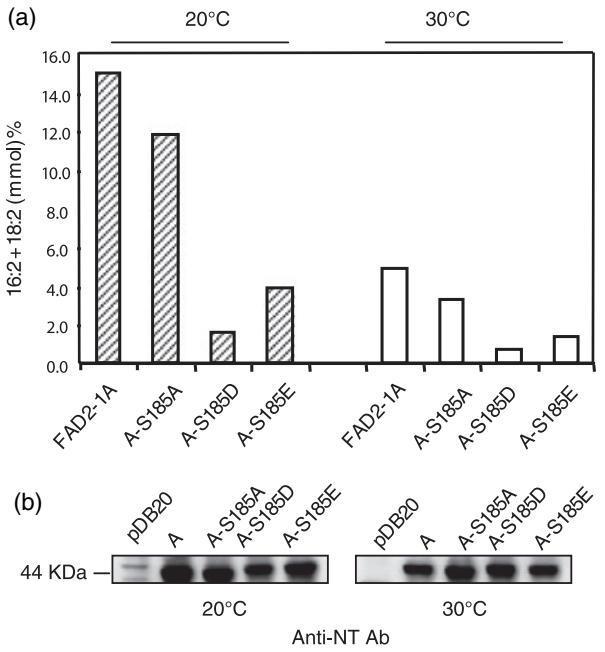

Figure 7. Expression in yeast of FAD2-1A constructs mutated at Ser[185].
(a) Dienoic fatty acid content of the yeast cells expressing the S185A, S185D and S185A mutations at 20 and 30°C.
(b) Immunoblot analysis of yeast cells expressing mutant FAD2-1A constructs using the Anti-NT antibody.

Discussion

Because membrane-bound fatty acid desaturases from plants have proven to be recalcitrant to purification and *in vitro* analyses (Browse *et al.*, 1993; Schmidt *et al.*, 1994), investigating their biochemical properties within their native environment remains a difficult problem. As an alternative means of overcoming this obstacle, the heterologous expression of these desaturases in yeast has been widely adopted (Broadwater *et al.*, 2002; Brown *et al.*, 1998; Bruner *et al.*, 2001; Cahoon and Kinney, 2004; Covello and Reed, 1996; Dyer *et al.*, 2001; Pirtle *et al.*, 2001). Yeast is an ideal background for the study of plant oleate desaturase genes because it provides a suitable membrane environment (the ER) and the requisite electron donor (cytochrome b_5), and lacks enzyme activities capable of synthesizing PUFAs. An important caveat to any study conducted in a heterologous system, however, is the fact that the function and properties observed in the non-native system may not be entirely recapitulated within its endogenous environment. Nevertheless, the observations derived from the expression of *FAD2-1A* and *FAD2-1B* in yeast should be useful in providing the initial foundation for understanding the mechanisms by which the stability and/or activity of these enzymes are regulated by temperature within the developing soybean seed.

Analysis of chimeric *FAD2-1A:FAD2-1B* constructs revealed two regions of the enzyme that appear to play an important role in the temperature-dependent stability of the enzymes. The N-terminal 44 aa of the two FAD2-1 enzymes and the region between positions 241 and 334 both differ at only four residues. The introduction of either segment from *FAD2-1A* into the *FAD2-1B* construct, however, resulted in a severe reduction in enzyme stability. The instability conferred by the N-terminal *FAD2-1A*-derived sequence shows similarity to the post-transcriptional mechanism of regulation described for the rat stearoyl-CoA

desaturase (SCD) enzyme. Unlike plant stearoyl-ACP desaturases which are soluble, plastid-localized enzymes (Shanklin and Cahoon, 1998), animal SCDs share features common to plant membrane-bound desaturases, such as its localization to the ER membrane, positioning of His-domains and dependence on cytochrome b_5 (Shanklin *et al.*, 1994). The first 33 aa of the rat SCD serves as a degradation signal, ensuring the rapid turnover of the enzyme (Mziaut *et al.*, 2000). The addition of the N-terminal 33 aa of rat SCD to a green fluorescent protein was sufficient to mediate the rapid turnover of this normally stable reporter protein. The N-terminal region of FAD2-1A may be viewed similarly as a temperature-dependent destabilization domain, given its ability to confer greater instability to the FAD2-1B enzyme at 30°C when these domains are substituted (Figure 3). However, it is clear that the impact of this domain cannot be interpreted simply as that observed with the rat SCD, as the reciprocal substitution of this region from FAD2-1B onto the FAD2-1A construct failed to overcome the temperature-dependent instability of the latter. Our results suggest a more complex model, where interaction among multiple domains is ultimately responsible for determining enzyme stability.

The enhanced stability of FAD2-1A at 30°C in yeast cells either treated with the inhibitor MG132 or possessing knockout mutations in certain UBC E2 genes clearly implicates the 26S proteasome in the temperature-dependent instability of the desaturase enzyme. Although the E3 ligases play the greatest roles in determining the substrate specificity of proteins degraded via the ubiquitin/26S proteasome pathway (Finley *et al.*, 2004), the UBC enzymes also serve to regulate the process by either determining specificity for particular classes of protein, mediating degradation in specific intracellular compartments, or controlling the process in response to developmental or environmental cues (Hampton and Bhakta, 1997; Jentsch *et al.*, 1990; Wiebel and Kunau, 1992). In our studies, expression of FAD2-1A in the UBC2 mutant background was particularly effective in enhancing enzyme accumulation and the dienoic fatty acid content at 30°C (Figure 5). Interestingly, UBC2 has been shown to be specifically involved in the ubiquitin-dependent N-end-rule pathway of protein degradation (Dohmen *et al.*, 1991; Madura *et al.*, 1993). In addition to our chimeric construct studies described above, this result appears to further implicate the N-terminus as being particularly important in mediating the temperature-dependent turnover of FAD2-1A.

The proper maintenance of membrane fluidity is a dynamic process largely dependent on the ability of an organism or cell to alter its membrane fatty acid composition in response to changes in environmental temperature. Given that the topographical characteristics of yeast membranes undoubtedly change as FAD2-1-derived dienoic fatty acids are introduced, it is tempting to speculate that this process is an integral part of the observed temperature-dependent reduction in FAD2-1 protein levels in yeast. For example, ubiquitin/26S proteasome-dependent degradation motifs that may be masked in an environment of reduced membrane fluidity may become revealed as fluidity increases due to the synthesis and incorporation of PUFAs. As PUFA levels increase, enhanced turnover of the FAD2-1 proteins would, in turn, lead to a reduction in PUFA synthesis. A similar model of feedback regulation has been proposed for plant FAD3 proteins (Dyer *et al.*, 2001). Decreased sensitivity of the FAD2-1B enzyme to alterations in membrane fluidity could thereby account for the establishment of a fatty acid equilibrium higher in PUFAs at higher temperatures than could be achieved by the more susceptible FAD2-1A isoform. Given the implications of such a model for our understanding of how soybeans modulate PUFA accumulation during seed development, it will be critical to establish the degree to which our yeast-based observations are recapitulated within the native plant environment.

In higher plants, a wide array of metabolic processes are regulated via protein phosphorylation. Our results suggest that the Ser residue at position 185 of the soybean FAD2-1 enzymes can indeed be phosphorylated, and that the protein kinase(s) responsible appears to be calcium stimulated (Figure 6c). Ser^{185} is predicted to reside at the ER:cytosol interface in close proximity to the active site of the enzyme (Figure 1b). It is therefore plausible that the introduction of a charged residue at this location would have an impact on enzyme activity. The substitution of an Asp or Glu residue at this position did in fact result in a reduction in enzyme activity (as evidenced by a decrease in dienoic fatty acid accumulation) when the mutated enzymes were expressed in yeast. Because the introduction of acidic residues at position 185 did not alter protein stability when the FAD2-1A enzyme was expressed in the heterologous system (Figure 7b), a reduction in enzymatic activity *per se* is the most likely explanation of the consequences of the substitution mutations.

The effects of FAD2-1 phosphorylation within its endogenous plant environment, however, is less clear. As inferred from the yeast expression studies, phosphorylation of FAD2-1 enzymes within the developing soybean seed may merely serve as a mechanism to downregulate its enzymatic activity. Phosphorylation-mediated inhibition of function represents one of the mechanisms by which the metabolic enzymes nitrate reductase and sucrose-phosphate synthase are known to be regulated (McMichael *et al.*, 1995). Another possibility is that phosphorylation may initiate degradation of the FAD2-1 enzymes in a manner similar to that demonstrated for the pyruvate kinase enzyme in developing soybean seeds (Tang *et al.*, 2003).

Judged by the representation of their respective ESTs within GenBank, it is apparent that both *FAD2-1A* and

FAD2-1B contribute toward soybean oil quality, although the latter may exert a greater influence given that more than twice as many *FAD2-1B* ESTs are found in comparison with *FAD2-1A* (Table 1). The constitutively expressed *FAD2-2* gene(s) would also be expected to contribute toward the overall oleate desaturase activity of the soybean seed. Because of the total number of *FAD2* genes involved, the differential stabilities of FAD2-1A versus FAD2-1B, and further potential of control via phosphorylation, it is likely that the regulation of 18:1 desaturation during triacylglycerol synthesis in soybean is complex. Thus it is not surprising that six independent quantitative trait loci (QTL) have been mapped that govern seed 18:1 content among different soybean genotypes and/or environments (http://soybase.agron.iastate.edu). In contrast to the complexity observed in soybean, the model plant Arabidopsis possesses a single *FAD2* gene (encoding an enzyme that lacks the Ser^{185} phosphorylation motif). In a recent analysis of recombinant inbred lines derived from two Arabidopsis ecotypes that differ significantly in oil content and composition, a single QTL was identified that associated with 18:1 content (Hobbs *et al.*, 2004). Not surprisingly, this QTL mapped to the same chromosomal region as the *FAD2* gene.

The value and specific applications of any vegetable oil are determined primarily by the unique fatty acid composition characteristic of the oilseed crop from which it was derived. The maintenance of an optimal fatty acid profile, however, can be greatly compromised by environmental factors, particularly temperature. Our study suggests that there are structural features of the FAD2-1B enzyme that allow it to be more stable than the closely related FAD2-1A isoform in response to changes in growth temperature. A more precise understanding of these motifs, and the mechanisms underlying the temperature-dependent degradation of FAD2-1 enzymes, may lead to the engineering of fatty acid desaturase enzymes that function more uniformly when exposed to changes in temperature and/or membrane fluidity.

In conclusion, the results of this study have provided new insights into the mechanisms by which oleate desaturase enzymes may be regulated. Subtle differences in amino acid sequence between the closely related FAD2-1A and FAD2-1B isoforms proved to be responsible for substantial differences in the temperature-dependent stability of the enzymes when expressed in yeast. Furthermore, the capacity of the FAD2-1 enzymes to be phosphorylated provides an additional avenue of regulatory control. The knowledge and tools acquired during this investigation will enable detailed investigation of the abundance, stability and phosphorylation status of the FAD2-1 enzyme within the plant, both throughout soybean seed development and in response to plant growth at varying temperatures.

Experimental procedures

Materials

All oligopeptides and peptide-specific antibodies were produced and affinity-purified by Bethyl Laboratories (Montgomery, TX, USA). The peptide sequences used were MGLAKETTMGGRGRVAKVEV for the Anti-NT antibody; DDTPYKALWREARE for the Anti-CT antibody; and KYLNNPLGRAVpS^{185}LLVTL for the Anti-pSer^{185} antibody. The ratio of specificity of the anti-phosphopeptide antibodies for the phosphopeptide antigen compared with the unphosphorylated sequence was >99:1 by ELISA (as determined by the manufacturer). Proteasome inhibitor MG132 was obtained from CalBiochem (La Jolla, CA, USA). *Saccharomyces cerevisiae* strain CTY182 (*MATa ura3-52*, *lys2-801*, *Δhis3-200*) was used for all yeast expression studies, with the exception of the UBC deletion studies which were conducted in strain BY4741 (*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*).

Soybean microsomal preparations for SDS–PAGE and immunoblot analysis

Frozen soybean seeds were powdered with liquid nitrogen using a mortar and pestle and mixed with 4 vol extraction buffer containing 50 mM 3-*N*-morpholinopropanesulphonic acid (MOPS)–NaOH pH7.5, 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 250 mM sucrose, and a cocktail of protease inhibitors [1 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride hydrochloride, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine HCl, 5 μM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E64), 50 μM leupeptin, 20 μg ml^{-1} soybean trypsin inhibitor and 5 mM ethylenediaminetetraaceticacid (EDTA)], phosphatase inhibitors (50 mM sodium fluoride and 1 μM microcystin-LR), and the proteasome inhibitor MG132 (10 μM). After centrifugation at 34 000 ***g*** for 10 min at 4°C, the supernatant was subjected to ultracentrifugation at 100 000 ***g*** for 1 h at 4°C. The resulting microsomal pellet was rinsed briefly three times and resuspended in 50 mM MOPS–NaOH, 2 mM DTT, 5 mM EDTA. Microsomal protein (10 μg) was separated on 15% sodium dodecyl sulphate (SDS) polyacrylamide gels, transferred to polyvinylidene difluoride membranes and bound to antibodies according to the protocols for the ECL Plus kit (Amersham Biosciences, Amersham, UK).

Construction of nested and mosaic FAD2-1 constructs

A PCR-mediated ligation strategy was used to generate chimeric FAD2-1 constructs as previously outlined (Nikawa and Kawabata, 1998; Vallette *et al.*, 1989) with some minor modifications. Pfu_{turbo} polymerase (Stratagene, La Jolla, CA, USA) was used for all PCR reactions. As an example, the nested construct AF_1BS_1 was generated by amplifying the 5′ region of the FAD2-1A cDNA (the AF_1 segment) with the forward primer, 5′-GAATTCAAGCTTATGGGTCTAGCAAAGGAAACAAC-3′ and the reverse primer, 5′-GTTTGGAACCCTTGAGAGAGG-3′. The FAD2-1B-derived segment (BS_1) of this construct was generated using 5′-CCTCTCTCAAGGGTTCCAAAC-3′ as the forward primer and 5′-GAATTCAAGCTTTCAATACTTGTTCCTGTACCAATAC-3′ as the reverse primer against a FAD2-1B template. The *Hin*dIII restriction sites engineered to facilitate the subsequent cloning of the construct are underlined. These initial PCR reactions were conducted by heat denaturing the DNA at 94°C for 2 min followed by 36 denaturation cycles at 94°C for 45 sec, annealing at 58°C for 45 sec, followed by extension at 72°C for 1.5 min. The AF_1 and BS_1 fragments were joined by combining

the individual amplification products in a second PCR reaction using the outermost primer pairs (5′-GAATTCAAGCTTATGGGTCTAGCAAAGGAAACAAC-3′ and 5′-GAATTCAAGCTTTCAATACTTGTTCCTGTACCAATAC-3′). PCR reaction conditions were as for the initial round, with the exception of a 2-min extension time. The integrity of the resultant chimeric product was confirmed by DNA sequence analysis. The PCR-mediated ligation product (AF_1BS_1) was digested with *Hin*dIII and inserted into the *Hin*dIII site of the yeast expression vector pDB20.

The remaining nested constructs were generated in an identical manner using the following primers: AF_2BS_2, 5′-GAATTCAAGCTTATGGGTCTAGCAAAGGAAACAACAATGGGAGGTAGAGG-3′ and 5′-CTTTCTTGAGTTGGCCAACAGTGAATGGTGGCTTTGTGTTTG-3′ (FAD2-1A template); 5′-AATTCAAGCTTTCAATACTTGTTCCTGTACCAATACACGCCCTTCTCGG-3′ and 5′-CCAAACACAAAGCCACCATTCACTGTTGGCCAACTCAAGAAAG-3′ (FAD2-1B template); AF_3BS_3, 5′-GAATTCAAGCTTATGGGTCTAGCAAAGGAAACAACAATGGGAGGTAGAGG-3′ and 5′-CCATGCAACTTTGGATTTTGGTTTTGGGACAAACACTTCATCAC-3′ (FAD2-1A template) and 5′-GTGATGAAGTGTTTGTCCCAAAACCAAAATCCAAAGTTGCATGG-3′ and 5′-CCAAACACAAAGCCACCATTCACTGTTGGCCAACTCAAGAAAG-3′ (FAD2-1B template); AF_4BS_4, 5′-GAATTCAAGCTTATGGGTCTAGCAAAGGAAACAACAATGGGAGGTAGAGG-3′ and 5′-GTCACAGAAAACAAAGCAACATCAGAGACATAGATCAAAAGCCTC-3′ (FAD2-1A template) and 5′-GAGGCTTTTGATCTATGTCTCTGATGTTGCTTTGTTTTCTGTGAC-3′ and 5′-CCAAACACAAAGCCACCATTCACTGTTGGCCAACTCAAGAAAG-3′ (FAD2-1B template); BF_1AS_1, 5′-GAATTCAAGCTTATGGGTCTAGCAAAGGAAACAATAATGGGAGGTGGAGG-3′ and 5′-CTTTCTTGAGTTGGCCAACAGTGAATGGTGGCTTTGTGTTTGG-3′ (FAD2-1B template) and 5′-CCAAACACAAAGCCACCATTCACTGTTGGCCAACTCAAGAAAG-3′ and 5′-CCAAACACAAAGCCACCATTCACTGTTGGCCAACTCAAGAAA-3′ (FAD2-1A template); BF_2AS_2, 5′-GAATTCAAGCTTATGGGTCTAGCAAAGGAAACAATAATGGGAGGTGGAGG-3′ and 5′-CCATGCAACTTTGGATTTTGGTTTTGGGACAAACACTTCATCAC-3′ (FAD2-1B template) and 5′-GTGATGAAGTGTTTGTCCCAAAACCAAAATCCAAAGTTGCATGG-3′ and 5′-CCAAACACAAAGCCACCATTCACTGTTGGCCAACTCAAGAAAG-3′ (FAD2-1A template); BF_3AS_3, 5′-GAATTCAAGCTTATGGGTCTAGCAAAGGAAACAATAATGGGAGGTGGAGG-3′ and 5′-GTCACAGAAAACAAAGCAACATCAGAGACATAGATCAAAAGCCTC-3′ (FAD2-1B template) and 5′-GAGGCTTTTGATCTATGTCTCTGATGTTGCTTTGTTTTCTGTGAC-3′ and 5′-CCAAACACAAAGCCACCATTCACTGTTGGCCAACTCAAGAAAG-3′ (FAD2-1A template); BF_4AS_4, 5′-GAATTCAAGCTTATGGGTCTAGCAAAGGAAACAATAATGGGAGGTGGAGG-3′ and 5′-TTGCATGGTAATGTGGCATTGTAGAGAAGAGATGGTGAGC-3′ (FAD2-1B template) and 5′-GCTCACCATCTCTTCTCTACAATGCCACATTACCATGCAA-3′ and 5′-CCAAACACAAAGCCACCATTCACTGTTGGCC AACTCAAGAAAG-3′ (FAD2-1A template).

The mosaic constructs $A_7B_8A_9$ and $B_7A_8B_9$ were constructed in a similar manner by combining the products of three individual PCR reactions. To construct $A_7B_8A_9$, the following primers were used: 5′-GAATTCAAGCTTATGGGTCTAGCAAAGGAAACAACAATGGGAGGTAGAGG-3′ and 5′-GTCACAGAAAACAAAGCAACATCAGAGACATAGATCAAAAGCCTC-3′ (FAD2-1A template); 5′-GAGGCTTTTGATCTATGTCTCTGATGTTGCTTTGTTTTCTGTGAC-3′ and 5′-TTGCATGGTAATGTGGCATTGTAGAGAAGAGATGGTGAGC-3′ (FAD2-1B template); 5′-GCTCACCATCTCTTCTCTACAATGCCACATTACCATGCAA-3′ and 5′-GAATTCAAGCTTTCAATACTTGTTCCTGTACCAATACACGCCCTTCTCGG-3′ (FAD2-1A template). To generate $B_7A_8B_9$ the following primers were employed: 5′-GAATTCAAGCTTATGGGTCTAGCAAAGGAAACAATAATGGGAGGTGGAGG-3′ and 5′-GTCACAGAAAACAAAGCAACATCAGAGACATAGATCAAAAGCCTC-3′ (FAD2-1B template); 5′-GAGGCTTTTGATCTATGTCTCTGATGTTGCTTTGTTTTCTGTGAC-3′ and 5′-TTGCATGGTAATGTGGCATTGTAGAGAAGAGATGGTGAGC-3′ (FAD2-1A template); 5′-GCTCACCATCTCTTCTCTACAATGCCACATTACCATGCAA-3′ and 5′-GAATTCAAGCTTTCAATACTTGTTCCTGTACCAATACACGCCCTTCTCGG-3′ (FAD2-1B template).

Yeast transformation, culture and microsomal preparations

Transformation and growth of yeast cells was conducted using standard protocols (Gietz *et al.*, 1995). To isolate microsomal fractions, 50-ml cultures of yeast cells grown in synthetic glucose minimal medium lacking uracil were harvested by centrifugation at 1000 ***g*** for 10 min and washed twice using 30 ml sterile water. The cell pellet was resuspended in 1 ml of the same extraction buffer described above for soybean microsomal extractions. Cells were broken using 0.45-mm glass beads and the FastPrep beadbeater (BIO 101, Vista, CA, USA) followed by a 1-min spin at 34 000 ***g*** at 4°C to remove the unbroken debris. The resulting supernatant (1 ml) was mixed with 4 ml of the same extraction buffer and the microsomal fraction was recovered by ultracentrifugation as described for the soybean microsomal preparations.

To inhibit protein synthesis, cycloheximide was added to 250 ml yeast cultures [OD = 1] at a concentration of 100 μg ml^{-1} (Gupta *et al.*, 2001). Aliquots (50 ml) were harvested according to the time course described in the text.

Partial purification of protein kinases from soybean seeds

Partial purification of protein kinases was performed as detailed by Tang *et al.* (2003). Briefly, frozen seeds (6 g) were homogenized in 50 ml extraction buffer containing 50 mM MOPS–NaOH pH7.5, 10 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride and 0.1% (v/v) TritonX-100 in a cold mortar. The homogenate was filtered through two layers of Miracloth (CalBiochem). Soluble proteins that precipitated from 5 to 15% (w/v) polyethylene glycol 8000 (PEG 8000) were collected by centrifugation at 34 000 ***g*** for 20 min at 4°C. The protein pellet was resuspended in 10 ml buffer A (50 mM MOPS–NaOH pH7.5, 10 mM $MgCl_2$, 2.5 mM DTT). After clarification by centrifugation at 34 000 ***g*** for 20 min at 4°C, the sample was applied to a 10-ml Resource Q anion-exchange column (Amersham Biosciences). After washing with buffer A, bound protein was eluted with an 80-ml linear gradient from 0 to 500 mM NaCl in buffer A at a flow rate of 1 ml min^{-1}. Active fractions were pooled separately and dialysed against buffer A without NaCl.

Peptide kinase assay

The peptide kinase assay was carried out in a 40-μl volume of 50 mM MOPS–NaOH pH7.5, 10 mM $MgCl_2$, 0.1 mM ATP ([γ-^{32}P] ATP 200 counts per minute $pmol^{-1}$), 0.1 mg ml^{-1} peptide, 0.1 mM $CaCl_2$ or 1 mM EGTA (as specified in the text) and containing 4 μl of a Resource Q column fraction. The reaction was initiated by addition of ATP and incubated for 10 min at room temperature. After incubation, 30 μl reaction mixture was spotted onto P81 phosphocellulose paper squares (4 cm^2). After three 10-min washes with 75 mM *o*-phosphoric acid to remove unincorporated γ-^{32}P ATP, the radioactivity on the paper square was detected by liquid scintillation counting.

Lipid analysis

Fatty acid methyl esters (FAMEs) of yeast samples were prepared directly from yeast pellets by acid methanolysis. Pellets were

suspended in 3 ml 5% HCl in methanol, transferred to 7-ml glass vials with teflon-lined caps, and incubated for 90 min at 85°C. After the samples were cooled to room temperature, 2 ml aqueous 1.5% NaCl (w/v) and 1 ml hexane were added sequentially. Samples were vortexed for 30 sec and centrifuged at 500 ***g*** for 5 min to separate phases. The upper hexane phase was aspirated and transferred to a 10 × 75-mm glass tube. A second addition, extraction and aspiration of hexane were then performed. Samples were evaporated to dryness at 40°C under a stream of nitrogen gas, redissolved in 1 ml hexane, and transferred to 12 × 32-mm crimp-top vials for analysis by gas chromatography (GC).

Yeast FAME analysis was performed using an Agilent Technologies Model 6890N GC (Palo Alto, CA, USA) and CHEMSTATION software. The analytical parameters were as follows. A 30 × 0.53-mm DB-23 column was used at 200°C with a 6.7 ml min^{-1} flow of He. The sample injector was at 250°C with a 10:1 split ratio and FAMEs were detected by a flame ionization detector (FID) operated at 300°C. Identities of unknown peaks were determined by retention-time comparison and calibration with an authentic FAME standard mixture (AOCS No. 6, Alltech Associates, Dearfield, IL, USA). For all constructs tested, lipid analysis was conducted on three independent yeast cultures.

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